Effect of Diet and Mating Status on Ovarian Development in a Predaceous Stink Bug *Perillus bioculatus* (Hemiptera: Pentatomidae)

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ABSTRACT A method is presented to quantitatively score the degree of ovarian maturation in a predacious pentatomid with asynchronous follicle development. The effects of artificial diet and mating status on ovarian maturation rates were examined. Ovarian scores were not influenced by mating status but were significantly lower in females fed the artificial diet. Ovarian follicles start forming when females are 2.3 d old, reaching a peak at 4.5 d. In controls, the rate of new follicle formation decreases after the onset of vitellogenesis. By 4.5 d, all control ovarioles contained at least 1 vitellogenic follicle, and by 9.5 d all ovarioles had chorionated follicles. In contrast, females fed the artificial diet had 40% of the ovarioles with a vitellogenic and chorionated follicle at 10.5 d of age. Mating started when females were 3.5 d old and correlated with the start of vitellogenesis. Two peaks in mating were observed, one at 4.5 d and the other at 9.5 d. Regulatory mechanisms for oogenesis are discussed.

KEY WORDS *Perillus bioculatus*, gonotrophic cycles, ovary scores

HEMIPTERA HAVE MEROISTIC telotrophic ovarioles (see Wigglesworth 1953), generally with 7 ovarioles per ovary (Carayon 1950). However, *Abedus ovatus* Stål, a belastomatid, has 5 ovarioles per ovary (Lalitha et al. 1997). In a telotrophic ovariole, the trophocytes are located in the germarium and are connected to oocytes via long nutritive cords (Woodruff and Anderson 1984, Huebner and Diehl-Jones 1993) rich in microtubules (Huebner and Diehl-Jones 1993, Lane and Stebbings 1998). When ovarian follicles become vitellogenic, the nutritive cord disconnects from the oocyte (Massner 1966, Brunt 1971, Lane and Stebbings 1998). Vitellogenin is produced by the fat body, moves through the interfollicular spaces, binds with its receptor on the oocyte plasma membrane, and is incorporated into the oocyte via receptor-mediated endocytosis (see Adams 1997, 1999a). Maximum growth of the ovarian follicle occurs in the vitellogenic phase of development. During vitellogenesis, a female may produce a batch of eggs that equals or exceeds her body weight at emergence. Therefore, if a female is to synthesize sufficient vitellogenin to guarantee a high level of fecundity, a nutritionally adequate diet is required.

Ovarian development has been divided into stages for several species of Hemiptera. Seven stages, based primarily on follicle cell morphology, were described for *Dysdercus fasciatus* Signoret (Brunt 1971), *Dysdercus koenigii* F. (Deshpande and Srivastava 1981) and *Abedus ovatus* (Lalitha et al. 1997). Patchin and Davey (1968) described 4 stages for *Rhodnius.* However, to determine the stages, the ovaries had to be sectioned and stained and in no case was an attempt made to quantify ovarian maturation in any of the Hemiptera examined to date.

Colorado potato beetles, *Leptinotarsa decemlineata* (Say), are major defoliators of potatoes worldwide (Hare 1990). The twospotted stink bug, *Perillus bioculatus* (F.) is a native North American predator (Knight 1952) of the Colorado potato beetle and has been used in augmentative biological control programs (Biever and Chauvin 1992, Hough-Goldstein et al. 1996). Before economically feasible augmentative releases of *Perillus bioculatus* are possible, a source of low-cost and high-quality insects must be available (Hough-Goldstein and Whalen 1993). One means of achieving this goal involves rearing the insects on cost-effective artificial diets. Diet development requires a rapid method to evaluate potential egg production, preferably during the midpoint of the first cycle of egg development. This article presents such a method based on easily determined ovarian follicle scores and is applicable to predaceous pentatomids used in biological control programs.

Materials and Methods

Rearing. Insects were reared in walk-in incubators held at 24.5 ± 0.5°C and 65% RH. The chambers were illuminated with high-pressure 400-W sodium lamps (#47–1481, Hummert International, Earth City, MO) from 0600 to 2100 hours. Twilight conditions were provided with 2 Sylvania Life Line F48-T12-CW-HO fluorescent bulbs per bank in 2 banks from 0500 to 0600 and 2100 to 2200 hours.

Perillus bioculatus eggs were obtained from Don C. Vacek's colony (USDA–APHIS–PPQ–MPPC, Mission TX) that originated from adults collected by K. D. Biever, Yakama, WA. At the time the colony was

established at Fargo, the insects had been reared for ≈40 generations.

Eggs (250–300) were placed in a white plastic tub 11.5 cm in diameter and 4.5 cm deep (TwinPak, Regina, Saskatchewan) with a water vial plugged with a cotton dental wick (TIDI Products, Troy, MI) and a potato leaf. The plastic tub was placed in a large cage. First and 2nd instars were given Colorado potato beetle eggs. Second through 5th instars and adults were provided with 5th-instar *Heliothis virescens* F. larvae. The caterpillars were frozen and stored at −25°C. Frozen larvae were placed in water and allowed to thaw in the refrigerator before placement in the cages. Second and 3rd instars were provided with 1 processed caterpillar daily. Third through 5th instars were given 7 processed larvae daily. Each cage contained 2 water vials that were changed every other day.

Adults were removed from the nymphal cages daily. An adult egging colony, consisting of 40 females and 10 males, was established in the large cages with the same set-up as for the nymphs, but the adults were provided with 10 processed caterpillars per day. Eggs were collected on Monday, Wednesday, and Friday and placed in the hatching tubs.

Cages. Small cages were made from 470 ml plastic wide-mouth jars (10 by 8.5 by 9.5 cm) (Consolidated Plastics,Twinsburg, OH), with a 3–210/36 Nitex screen (Tetko, Lancaster, NY) covering a hole (6.5 cm diameter) in the cap. Brown wrapping paper was cut into strips (6 cm by 0.5 m) and rolled up and placed in the cages to provide resting places. Large cages were constructed from Rubbermaid Hi Top storage boxes (Wooster, OH) that are 34 by 25 by 11 cm with a Nitex screen-covered 11-cm square hole in the lid. Each cage contained 4 rolls of coiled brown wrapping paper strips (6 cm by 1 m). Both the small and large cages had the uppermost portion of the inside surface coated with a 2-cm band of Fluron AD-1 (Northern Products, Woonsocket, RI) to keep the early instars off of the lid.

Analysis of Ovarian Maturation. A female was pinned ventral side up in paraffin-covered petri dish containing *Manduca* saline (see Riddiford et al. 1979). An incision was made laterally at the junction of the dorsal tergites and ventral sternites from anus to thorax. A cut was then made across the sternites and the entire ventral sternite section was removed. The reproductive system tends to adhere to the sternites. One ovary was removed, placed on a microscope slide in a drop of saline, teased apart to expose the individual ovarioles, and examined with a dissecting microscope at 10–40×. All follicles within each of the 7 ovarioles were scored as follows: 0, no observable follicle; 1, previtellogenic follicle; 5, vitellogenic follicle; and 10, mature chorionated follicle. Vitellogenic follicles contained yellowish colored yolk granules. The scores for follicles within each ovariole were totaled and the totals for the 7 ovarioles were summed to give a total score for the ovary. If a female was ≥5 d old and had a score of 7, the data were not included in the calculations because it was assumed either that these females would not develop their ovaries or development was retarded. Instead, the percentage of females with undeveloped ovaries at these ages was calculated.

The number of previtellogenic, vitellogenic, and mature follicles within ovarioles was recorded for ovaries in the experimental groups at different ages after emergence. The number of gonotrophic cycles and new follicle formation from the germarium at different ages also was calculated. Residues within the ovarioles also were recorded. Follicle cell residue, referred to as the corpus luteum, at the ovariole base and within the ovarian pedicel indicates that oviposition has occurred (Barth 1973). Follicle residues between follicles indicate that follicles have been resorbed, an irreversible degeneration of vitellogenic follicles.

Analysis of Mating. In the experiments where females were held with males, spermathecae were removed, placed in a drop of *Manduca* saline on a microscope slide and then crushed with a cover slip. The spermathecal preparation was then examined with phase contrast microscopy at a magnification of 160 x for the presence of sperm in the spermatheca bulb or duct. Samples of 5–12 spermathecae were taken at daily intervals from a starting population of 66 females. The percentage mating within a 24-h interval, MI, was calculated from the equation $MI = M_{t+1} - M_t$, where M_{t+1} is the percentage mating at day plus 1 and M_t is the percentage mating on the previous day.

New Follicle Formation. The number of new follicles formed during a 24-h interval, FI, was calculated in the same manner as mating during an interval from the equation: $FI = F_{t+1.} - F_t$, where F_{t+1} is the mean number of follicles per ovary at day plus 1 and F_t is the mean number of follicles on the previous day.

Artificial Diet. Artificial diet was prepared from the recipe provided by Tom Forrester (USDA–APHIS–PPQ–MPPC, Mission TX). A stock solution vitamin mix was prepared: water (200 ml), niacin (1.6 g), D-Ca pantothenic acid (1.6 g). Water (150 ml), sucrose (5 g), sodium benzoate (0.5 g), methyl paraben (1 g), and citric acid (1 g) were placed in a blender and mixed at low speed. Frozen chunks (227 g) of pork liver were added to the blender and mixed at high speed until smooth in appearance. Vitamin solution (1 ml) and hamburger (227 g) containing 25% fat were added and blended at high speed for 2 min. The diet mix was placed in clear plastic cups (No.410, Fill-Rite, Newark, NJ) and stored frozen at −70°C. A sheet of Parafilm M (American National Can, Greenwich, CT) was placed on the top of a Teflon test tube holder with 14-mm-diameter holes and pressed into the holes with the end of a rubber pipette suction bulb to produce domes. Artificial diet was thawed and introduced into the domes with a syringe and then the bottom of the Parafilm sheet was sealed with a sheet of Scotch Brand transparent tape (10 cm wide) (3 M, St. Paul, MN). The artificial diet packets were stored at 25°C until used. Packets were prepared weekly.

Experimental Conditions. Females (7) were held with either 3 males or without males from emergence to the time of sampling to evaluate possible mating effects on the rate of ovarian maturation. Another

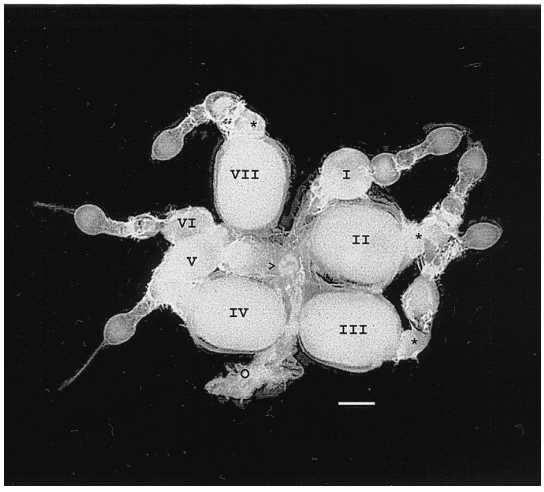

Fig. 1. A 14-d-old *P. bioculatus* ovary teased apart to expose the 7 ovarioles. I to VII, ovarioles; *, resorbed follicle; >, corpus luteum; O, lateral oviduct. Scale line = 0.5 mm.

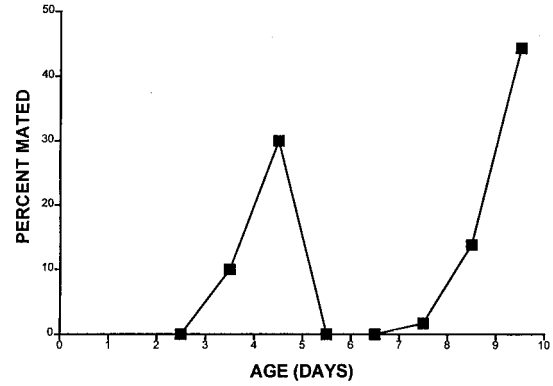

Fig. 2. Percentage of females that mated within a 24-h interval.

experiment quantified the effect of artificial diet on ovarian maturation in females held with males. Samples were taken daily at 0700 hours (age = day 0.5 ± 0.5) to give ages of 0.5–9.5 or 10.5 d after emergence. Data were analyzed with PC SAS (SAS Institute 1987)

Results

Description of Oogenesis. A single ovary from a 14-d-old prey-fed female from a colony cage was dissected to expose the 7 ovarioles (Fig. 1). Residue in the common oviduct indicated that this female had oviposited. Also, 3 ovarioles contained resorbed follicles. Ovarioles are numbered from I to VII and the ovary had a score of >50 (several follicles in IV were not scored because they could not be seen in the figure.). The largest follicles were in late vitellogenesis and had not started chorion formation.

Follicle scores also are correlated with follicle dimensions. Follicles with a score of 1 are usually spherical with diameters ranging from 230 to 490 μ (n = 15 follicles from different females). A score of 5 is associated with elongated follicles with lengths of 410–1,410 μ and widths of 250–940 μ (n = 11 follicles from different females). Follicles with scores of 10 have lengths of 1,380–1,620 and widths of 1,090–1,250 μ (n = 11 follicles from different females).

Mating. Mating was first observed in 3.5-d-old females (10%, n = 10) with the highest percentage (100%, n = 5) of mated females being found at 9.5 d. Between 3.5 and 4.5 d, 40% of the females had mated (Fig. 2). Another large increase of 60% occurred between 8.5 and 9.5 d. Thus, mating occurred in 2 peaks, one at 3.5–4.5 d and the other at 8.5–9.5 d. Mating correlated quite well with ovarian scores. Only 1 female with a score of 7 was mated (n = 25), whereas 80% of those with scores >112 were mated (n = 10). Twenty-seven percent of the females with scores between 8 and 42 (n = 11) and 63% between 43 and 112 (n = 30) were mated. Of the 25 females with scores of 7, half were older than 4.5 d, indicating that some factor influencing ovarian maturation, but not chronological age, might trigger female mating receptivity.

Ovarian Development. Virgin females or females held with males and fed the control diet had ovarian scores that were not significantly different from each other, but females given the artificial diet had significantly lower scores (treatment $P \leq 0.0001$; df = 2, 213; $P \leq 0.05$, Student–Newman–Keuls mean separation). Virgin females and those held with males had mean scores of 59.8 versus 63.3, respectively, compared with mean scores of 18.8 for females given the artificial diet.

Because there were no significant differences between the 2 groups of females provided with the control diet, the data for virgins and females held with males were combined and fit to a linear regression model (Fig. 3A). Ovarian scores fit the equation: score = 18.39 X − 42.21 with r = 0.79 (n = 177 points) and an x-intercept of 2.3 d. Ovarian scores for females provided with artificial diet did not fit a linear model (Fig. 3B), but fit a quadratic equation: score = 19.34 – 8.63 X + 1.06 X^2 with a correlation coefficient of 0.56 (n = 73 points). The average score for 9.5-d-old controls was 146 compared with 52.9 for 10.5-d-old females fed the artificial diet. Furthermore, the correlation coefficients, r, indicate that the insects given the artificial diet had much greater variation in ovarian scores than the controls. Thus, ovarian scores showed that insects fed the artificial diet developed ovaries at a much slower rate and had lower scores than those fed the control diet.

Follicle Residues and Nondeveloping Follicles. Resorbed follicles were found only in the 7.5-d control sample. Two of 24 females had a total of 3 resorbed follicles. No resorbed follicles were found in the insects fed the artificial diet. A sample of four 49-d-old females taken from a colony cage showed that each female contained ovaries with at least 1 resorbed follicle per ovariole. Thus, follicle resorption appears to be age-related.

Stunted ovaries, those with a score of ≤7, had a mean occurrence of 15.4 ± 6.5% in the controls at ages

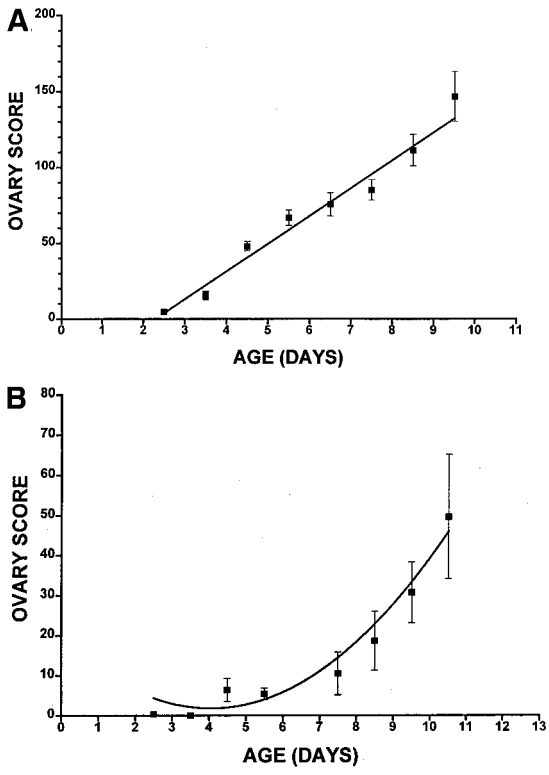

Fig. 3. Ovarian scores for *Perillus* at different ages. Error bars represent 95% confidence intervals. (A) Scores from pooled samples of virgins and females held with males. Regression line is based on 177 observations with 11–29 observations per point. (B) Score for females fed artificial diet. Regression line is based on 73 observations with 9–16 observations per point.

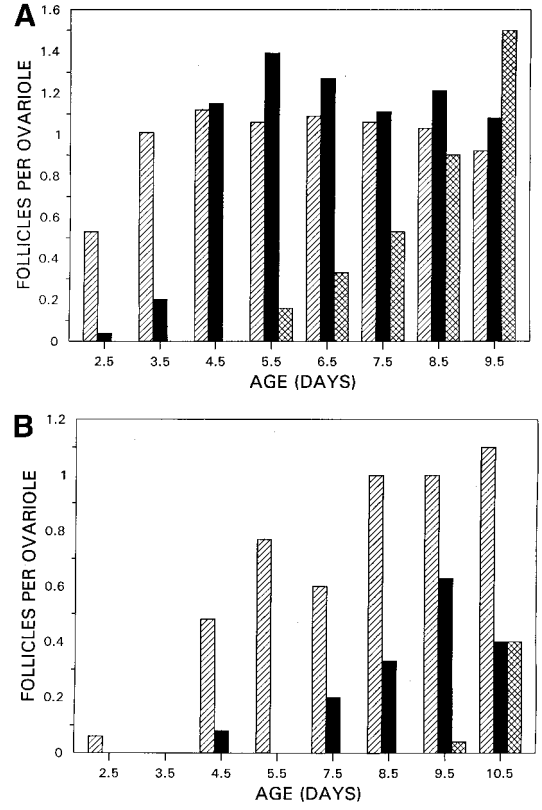

Fig. 4. Average number of follicle types within an ovariole of *Perillis* at different ages. (A) Pooled samples of virgins and females held with males. (B) Samples from females given artificial diet. Right hatch, previtellogenic follicles; solid, vitellogenic follicles; cross hatch, mature follicles. Calculated from data set in Fig. 3.

of 4.5–9.5 d. No apparent trend with age was observed in the data except that the 9.5-d-old females had the lowest percentage (6.7%) of nondeveloping ovaries, suggesting that the population contains females with different rates of ovarian maturation. Females given the artificial diet contained an average of 72.7± 22.5% stunted ovaries at ages of 4.5–10.5 d. However, these data showed a decrease in frequency of nondeveloping ovaries with female age from 100% in 5.5-d-old females to 54.5% in 10.5-d-old females.

Follicle Stage Distribution. The average distribution of follicles with scores of 1 (previtellogenic), 5 (vitellogenic), or 10 (mature follicle) within ovarioles for females of different ages is shown in Fig. 4. Only the germaria without any follicles in the ovarioles (score 0) were found in controls sampled at 0.5 and 1.5 d after emergence (Fig. 4A). By 4.5 d, each ovariole also had at least 1 vitellogenic follicle and by 9.5-d, all ovarioles contained at least 1 mature follicle. In contrast, females fed the artificial diet (Fig. 4B) started to develop vitellogenic follicles at 4.5 d and mature follicles first appeared at 10.5 d. Thus, oogenesis was retarded in insects given the artificial diet.

Variations in inter-and intraovariole ovarian development is demonstrated by scores for the 7 ovarioles (I–VII) within 1 ovary of a 9.5-d-old female fed the control diet as follows: I - 1, 5, 10; II - 1, 1, 5, 10, 10; III - 1, 5, 10; IV - 1, 5, 10; V - 1, 5, 5; VI - 1, 5, 10; VII - 1, 5, 10, 10. These follicle scores show that there is no correlation between the development of follicles either within or between ovarioles. Even the number of follicles present per ovariole varied from 3 to 5. Furthermore, >1 vitellogenic follicle or mature follicle may be present in an ovariole at any given time. Thus, ovarian development in *P. bioculatus* is asynchronous.

Gonotrophic Cycles. The average number of gonotrophic cycles present in an ovariole was not statistically different in females held with males or virgins when fed the control diet, but were significantly lower in females given the artificial diet. Pooled data for virgins and females held with males showed an increase of 0.85 follicles per day from 2.5 to 4.5 d after emergence to give an average number of 2.2 ± 0.4 cycles per ovariole (n = 19) by 4.5 d (Fig. 5A). However, from 4.5 to 9.5 d, new follicles were formed at the rate 0.21 follicles per day, resulting in 3.5 ± 0.8 cycles per ovariole (n = 11) by 9.5 d after emergence. The largest number of gonotrophic cycles observed was 5.3 per ovariole in a 9.5-d-old female that had 17 mature

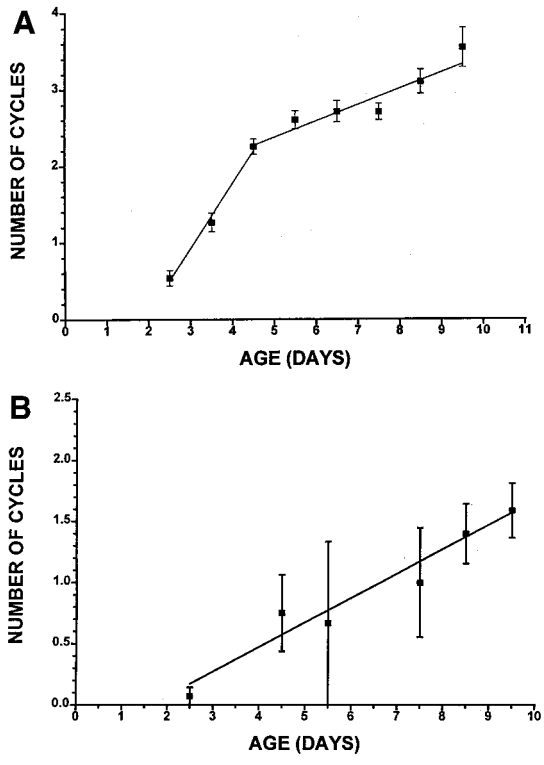

Fig. 5. Average number of gonotrophic cycles present in a *Perillus* ovariole at different ages. Error bars represent 95% confidence intervals. (A) Cycles from pooled samples of virgins and females held with males. (B) Cycles from females given artificial diet. Calculated from data set in Fig. 3.

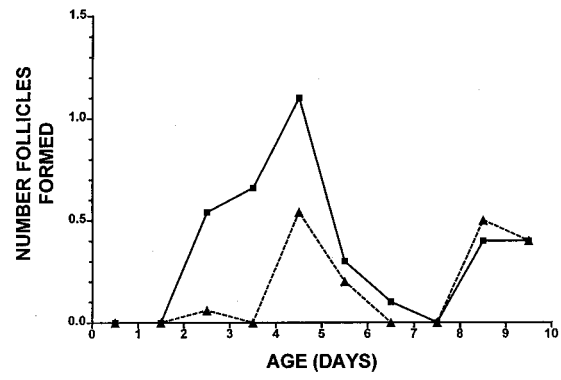

Fig. 6. Number of new follicles formed within a 24-h interval. Solid line, pooled samples of virgins and females held with males; dashed line, samples from females given artificial diet.

follicles, 13 vitellogenic follicles, and 7 previtellogenic follicles in 1 ovary.

Females given artificial diet exhibited an increase in the number of gonotrophic cycles of 0.2 cycles per day from 2.5 to 10.5 d after emergence (Fig. 5B). By 10. d, there were 1.7 ± 0.8 gonotrophic cycles per ovariole (n = 12). Thus, females given the artificial diet had 50% fewer gonotrophic cycles per ovariole than controls of the same age.

Changes in the number of follicles formed per ovariole in controls and those fed the artificial diet were calculated for 0.5–9.5 d of age at daily intervals (Fig. 6). A distinct peak in the number of follicles formed was observed for the 3.5–4.5-d interval in females fed either the control or artificial diet. A smaller peak was observed in the 7.58–8.5-d interval.

Discussion

Female *P. bioculatus* generally initiate mating at the start of vitellogenesis, and mating continues periodically throughout the lifetime of the female (unpublished data). During the development of the first batch of eggs, most matings occur between 6.5 and 9.5 d (Fig. 2). In some insects, endocrines induce female mating behavior and pheromone synthesis (Ringo 1996). At the present time, the inducers of female mating behavior in *Perillus* are not known, but could involve juvenile hormone because it is required for vitellogenesis in Hemiptera (see Adams 1997; 1999a; Davey 1997) and females with previtellogenic ovaries tend not to mate.

In several hemipterans, mating is required for the production of mature ovarian follicles. *Cimex lectularius* L. will develop follicles to vitellogenesis, but will resorb them if not mated (Davis 1956). Mating accelerates the rate of ovarian development in *Dysdercus fasciatus* Signoret (Brunt 1971). However, mating is not a prerequisite for oogenetic development in *Perillus.* There was no difference in ovarian scores between females held with males or virgins. The first cycle of ovarian maturation in *Rhodnius prolixus* (Ståhl) (Davey 1965; Davey et al. 1986), *Lygocoris pabulinus* L. (Wightman 1973), and *Adelphocoris lineolatus* (Goeze) (Masner 1966) also was independent of mating.

Follicle degeneration may occur in response to nutritional stress, aging, or blocking of oviposition and is a means of recycling nutrient-rich materials found in the oocyte (Bell and Bohm 1975). In *P. bioculatus,* the highest number of resorbing follicles was observed in older vitellogenic females, as it was in *A. lineolatus* (Masner 1966). This might be attributed to disruption of the processes involved in vitellogenin production and uptake.

Perillus bioculatus may have >1 vitellogenic follicle per ovariole (Fig. 4). *Adelphocoris lineolatus* (Goeze), a plant feeding mirid, typically contains 2 vitellogenic follicles per ovariole (Masner 1966). *Dysdercus* may contain up to 4 vitellogenic follicles per ovariole (Brunt 1971). This contrasts with *Rhodnius* where only 1 vitellogenic oocyte is present in an ovariole at any one time (Friend et al. 1965) because of the presence of an antigonadotropin (see Davey 1993, 1996, 1997; Adams 1999b). *Cimex lectularius* L. has reduced the number of mature follicles found in an ovary at any time to 1 (Davis 1956). Thus, ovarian maturation within Hemiptera follows no set pattern.

New follicles form in the ovarioles of *Perillus* between 1.5 and 7.5 and 7.5 and 9.5 d with a peak of follicle formation at 4.5 d (Fig. 6). The factors regulating follicle formation in *P. bioculatus* are not known, but in *Aedes aegypti* L., new follicle formation is induced by physiological levels of 20-hydroxyecdysone (Beckmeyer and Lea 1980). However, there is an interesting correlation between the presence of vitellogenic follicles in all ovarioles at 4.5 d in the controls (Fig. 4A) and a decrease in new follicle formation (Fig. 5A and 6). This correlation is not present in females fed the artificial diet.

Rhodnius ovarian maturation is characterized as having intraovariole synchrony because development within ovarioles, but not between ovarioles, is synchronized (see Adams 1997, 1999a, b). However, *P. bioculatus* develops ovaries asynchronously as does *Lygus lineolaris* (Palisot de Beauvois) (Ma and Ramaswamy 1987) and *Riptortus clavatus* Thunberg (Numata and Hidaka 1982).

Various schemes have been used to score hemipteran ovarian follicle development (Patchin and Davey 1968, Brunt 1971, Lalitha et al. 1997, Deshpande and Srivastava 1981), but none is quantitative or rapid. The method presented for scoring *Perillus* ovarian development is quantitative, rapid, and is the only method able to give an ovarian score to insects with asynchronous follicle development.

In *Rhodnius*, ovarian maturation is induced by a massive blood meal taken at monthly or greater intervals that induces an endocrine cascade resulting in ovarian maturation (see Davey 1997, Adams 1999a). The single blood-meal taken by *Rhodnius* provides sufficient nutrients for the development of 1 batch of eggs (Buxton 1930). *Perillus*, in contrast, feeds continuously and one would expect vitellogenic percursors to be available at all times, making it possible to develop >1 vitellogenic follicle per ovariole and to have vitellogenic follicles present continuously in the ovarioles in females ≥4.5 d old. The differences in hemipteran feeding strategies may explain the differences observed in egg development.

Several factors may be responsible for decreased ovarian scores and lowered fecundity (Rojas et al. 1999) in *Perillus* fed artificial diet (Fig. 3B). One may involve juvenile hormone titers because juvenile hormone is required for both vitellogenin synthesis and uptake (see Dittmann and Biczkowski 1995; Adams 1997, 1999a; Davey 1997). The other may involve lower vitellogenin levels caused by a paucity of vitellogenic precursors such as lipids, carbohydrates, or amino acids. Further work is required to determine which factor is involved in *Perillus* fed the artificial diet.

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